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### ABSTRACT
Several recent studies have indicated that decreased levels of the MCM2-7 DNA replication proteins can lead to genomic instability (GIN) and cancer formation. Interestingly, genetic or RNAi-mediated depletion of one MCM has been demonstrated to cause decreases in other MCMs, presumably as a consequence of MCM heterohexamer destabilization. In the first year of my training grant, my research results show that in cells bearing only the Mcm4\textsuperscript{Chaos3} cancer susceptibility allele, the cause for reduced MCM protein levels is related to decreased Mcm2-7 and 10 mRNA. Despite being present at levels far exceeding that required for DNA replication under normal circumstances, we found that heterozygosity for 2 or more different MCMs caused genomic instability, and in the cases of MCM2, MCM6 and MCM7, synthetic lethality in conjunction with Mcm4\textsuperscript{Chaos3} homozygosity. These data suggest that proper stoichiometry of MCM components is carefully regulated, and that relatively minor disregulation or destabilization of MCM levels can have serious consequences for survival or cancer susceptibility in whole animals.

### SUBJECT TERMS
MCM2-7, Genomic instability, DNA replication, Cancer susceptibility
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>5</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>8</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>9</td>
</tr>
<tr>
<td>Conclusion</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>11</td>
</tr>
<tr>
<td>Supporting Data</td>
<td>12-14</td>
</tr>
</tbody>
</table>
Introduction:

In eukaryotes, DNA synthesis begins at multiple sites along chromosomes called replication origins. In late mitosis to early G1 phase, replication origins are selected and bound by the hexameric origin recognition complex (ORC). ORC then recruits the initiation factors CDC6 and CDT1, which in turn are required for loading of the MCM2-7 complex, thereby forming the “pre-replicative complex” (pre-RC). The formation of pre-RCs is termed origin “licensing” and this allows origins to gain competency for a single round of DNA synthesis before entering S phase. MCM2-7 is a hexamer of six distinct but structurally-related minichromosome maintenance (MCM) proteins. In vivo and in vitro evidence indicates that the MCM2-7 complex is the replicative helicase (Bochman & Schwacha, 2008; Labib et al, 2000; Moyer et al, 2006).

MCM2-7 proteins exist abundantly in proliferating cells, and are bound to chromatin in amounts exceeding that which is present at active replication origins or which is required for complete DNA replication. Although many studies showed that drastic decreases in MCMs are tolerated by dividing cells, there are certain deleterious consequences. A reduction in MCM proteins reportedly causes decreased usage of certain ARSs (Lei et al, 1996), precipitating genome instability in yeasts. More recently, it was found that in Xenopus extracts and mammalian cells, excess chromatin-bound MCM2-7 complexes occupy dormant or "backup" origins that are activated under conditions of replication stress, compensating for stalled or disrupted primary replication forks. A depletion of these backup licensed origins could potentially lead to incomplete DNA replication and genetic aberrations (Ge et al, 2007; Ibarra et al, 2008; Woodward et al, 2006).

In previous work, Shima et al found that a hypomorphic allele of mouse Mcm4 (Mcm4<sup>chaos</sup>) caused high levels of GIN and extreme mammary cancer susceptibility. The ethylnitrosourea (ENU)-induced Mcm4<sup>chaos</sup> point mutation changed PHE to ILE at residue 345 (Phe345Ile). This amino acid is conserved across diverse eukaryotes and is important for interaction with other MCMs. Subsequently, it was reported that mice containing 1/3 the normal level of MCM2 succumbed to lymphomas at a very young age, and had diverse stem cell proliferation defects (Pruitt et al, 2007). These mice also had 27% reduced levels of MCM7 protein. Working with human cultured cells, Ge et al reported that Mcm5 knockdown decreased the amount of chromatin-bound MCM2,3,6 and 7 (total MCM2 was unchanged; levels of the others were not reported (Ge et al, 2007)), and Ibarra et al found that RNAi-mediated knockdown of Mcm2 or Mcm3 caused a drastic decrease in the levels of all other MCM2-7 proteins. They speculated that
elimination of a single MCM component destabilized the hexamer (Ibarra et al, 2008). In both cases, GIN and proliferation defects were observed under conditions of chemically-induced replicative stress. These studies suggest that in addition to the biochemical consequences of the Phe345Ile alteration, the overall decrease in MCM levels might contribute to GIN and cancer in \( \text{Mcm4}^{\text{Chao3}} \) mice. It also implies that relatively modest decreases in any of the MCMs may be sufficient to cause these and other developmental defects, such as those that affect stem cells in MCM2-deficient mice (Pruitt et al, 2007).

In my first year of funding, I have shown that genetically-induced reductions of MCM levels, achieved by breeding combinations of MCM2-7 alleles, can have severe consequences for embryonic development and cancer susceptibility in mice. Additionally, I found that pan-reduction of MCM levels in \( \text{Mcm4}^{\text{Chao3}} \) cells is caused posttranscriptionally. This suggests the existence of a novel regulatory relationship for governing the stoichiometry of the MCM DNA replication licensing complex.

Body of annual summary:

Based on my proposal and statement of work, I list all of the tasks and the progress during this year.

**Task 1: Determine the causes and mechanisms of genome instability in \( \text{Mcm4}^{\text{Chao3}} \) mice and cells**

In previous work, Shima et al found that a hypomorphic allele of mouse \( \text{Mcm4} \) (\( \text{Mcm4}^{\text{Chao3}} \)) caused high levels of GIN and extreme mammary cancer susceptibility. Surprisingly, MEFs from \( \text{Mcm4}^{\text{Chao3}} \) mice not only had reduced levels of MCM4, but also MCM7. To extend previous findings and to determine if the decreased levels were differentially compartmentalized in the cell, I quantified cytoplasmic and chromatin-bound MCM2-7 levels in mouse embryonic fibroblasts (MEFs) by Western blot analysis. As shown in Fig 1, all MCMs were decreased in both compartments by at least 40% compared to WT cells. These data suggest that levels of MCM2,3,5,6, & 7 were adjusted to the mutant \( \text{Mcm4}^{\text{Chao3}} \) allele in a similar manner, and that the cell does not appear to compensate for lack of chromatin-bound or cytosolic MCMs by shuttling between the two compartments (Fig 1).

Shima et al hypothesized that MCM4\(^{\text{Chao3}}\) might be unstable, causing dissociation of MCM2-7 hexamers and subsequent degradation of the individual proteins (Shima et al,
A similar argument was made to explain pan-MCM decreases in human cell cultures depleted of *MCM2* or *MCM3* by siRNA (Ibarra et al, 2008). If this were indeed the case, then we would expect mRNA levels to be unchanged in mutant cells. To measure the amounts of *Mcm2-7* mRNA in *Mcm4^Chao3/Chao3* MEFs, I performed quantitative RT-PCR (qRT-PCR) analysis of these genes, *Mcm10*, and several control housekeeping genes. Analysis of 5 littermate pairs of primary MEF cultures (pairs were derived from littermates in all cases) revealed that transcript levels for each of these genes in mutant cells was 51-65% of WT, in line with that of protein decreases (Fig. 2a). These mRNA decreases were specific to the Mcms, as the 7 housekeeping genes analyzed were not altered significantly (Fig. 2b). These data suggest that the decrease of MCM proteins in *Mcm4^Chao3/Chao3* cells is the result of decreased mRNA.

The reduction of Mcm mRNAs could be due to decreased transcription or increased posttranscriptional degradation. To test the former, I used quantitative real-time RT-PCR (qRT-PCR) to measure the levels of heterogeneous nuclear RNA (hnRNA; pre-spliced transcripts), which, due to its short half-life, reflects transcriptional activity. In contrast to the mRNA (Fig. 2), Mcm hnRNA levels in *Mcm4^Chao3/Chao3* and WT MEFs were approximately the same (Fig. 3a), implying that post-transcriptional elimination or destabilization of MCM mRNAs underlies the Mcm mRNA and protein decreases. However, it is conceivable that transcription is decreased but splicing is inhibited proportionally, which would lead to apparently normal hnRNA levels but decreased mRNA.

To address this, I performed two experiments to examine Mcm transcriptional activity. First, I measured RNA polymerase II occupancy within Mcm transcriptional units by chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR). The levels in mutant cells were similar to or higher than in WT (Fig. 3b). In a second approach, I cloned the putative promoter regions (-1 to -300 bp) of *Mcm2, Mcm5*, and *Mcm7* upstream of a firefly luciferase reporter, transfected these transiently into WT and *Mcm4^Chao3/Chao3* MEFs, and measured luciferase levels. There were no significant differences between control and WT cells for any of the promoters (Fig. 3c). The combined data indicate that the lower Mcm mRNA levels in *Chao3* cells are not due to decreased transcription.

Task 2: Determine if decreased levels of various MCM proteins cause genomic instability (GIN) and cancer predisposition in mouse models.

The *Mcm4^Chao3* allele was identified in a forward genetic screen for mutations causing elevated micronuclei (MN) in red blood cells, an indicator of GIN (Shima et al, 2007a).
2007a). While the altered MCM4\textsuperscript{Chaos3} protein probably causes replication errors as does a yeast allele engineered to contain the same amino acid change (Li et al, 2009), it is also possible that the decrease in overall MCM levels in Mcm4\textsuperscript{Chaos3} mutants leads to elevated S-phase DNA damage and GIN. To test this possibility, I generated mice from ES cells bearing gene trap disruptions of Mcm2, Mcm3, Mcm6, and Mcm7 (alleles are designated as Mcm\#Gt), bred them into the C3HeB/FeJ genetic background for at least 4 generations (Mcm4\textsuperscript{Chaos3}/Chaos females get mammary tumors in this background), and measured MN levels. As with a previously reported Mcm4 gene trap allele (Shima et al, 2007a), each of these alleles proved to be recessive embryonic lethal (Table 1). Heterozygosity for each gene trap allele caused elevated MN (Fig. 4). Compound heterozygosity further increased MN on average, as did heterozygosity for 3 or more gene traps (Fig. 4).

Previous studies showed that reductions of particular MCMs in cells or mice reduces the levels of other MCMs, causing GIN, cancer, and developmental defects. However, the reduction in MCM levels required to precipitate these consequences, and whether there is a threshold effect, is unclear. For example, the early-onset lymphoma and stem cell deficiency phenotypes in mice with a hypomorphic MCM2 allele (Mcm2\textsuperscript{RES-CreERT\textsuperscript{2}}) occur in homozygotes having 35% of WT MCM2 protein, but not heterozygotes (62% of WT) (Pruitt et al, 2007). Cells from these homozygotes also showed a 21% reduction of MCM7. C3H-\textit{Mcm4}\textsuperscript{Chaos3/Chaos} mice are developmentally normal, but Mcm4\textsuperscript{Chaos3/-} animals die in utero or neonatally depending on genetic background (Shima et al, 2007b). These cases support the idea that there is a replication licensing threshold effect with respect to the cancer and developmental phenotypes.

To explore the consequences of incremental MCM reductions on viability and cancer, I introduced the Mcm4\textsuperscript{Chaos3} and Mcm2\textsuperscript{Gt} alleles into the same genome. The most striking was a highly significant shortfall of Mcm4\textsuperscript{Chaos3/Chaos} Mcm2\textsuperscript{Gt/+} offspring at birth. Heterozygosity for Mcm2\textsuperscript{Gt} itself was not haploinsufficient [Mendelian transmission of Mcm2\textsuperscript{Gt} in crosses of heterozygotes to WT (119/250; $\chi^2 = 0.448$)]. These results demonstrate that there is a synthetic lethal interaction between Mcm4\textsuperscript{Chaos3} and Mcm2\textsuperscript{Gt}, thus suggesting that modest decreases in MCM levels can compromise embryonic development.

The synthetic interaction between Mcm4\textsuperscript{Chaos3} and Mcm2\textsuperscript{Gt} might be specific, or it may reflect a general consequence of reduced replication licensing (and consequent elevated replication stress). I therefore tested whether hemizygosity for Mcm6 or Mcm7 would also cause synthetic phenotypes in an Mcm4\textsuperscript{Chaos3/Chaos} background. The Mcm4\textsuperscript{Chaos3/Chaos} Mcm6\textsuperscript{Gt/+} genotype caused highly penetrant embryonic lethality; only
10% of the expected number of such animals survived to birth. The \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) \( \text{Mcm7}^{\text{Gt1+}} \) genotype caused both embryonic and postnatal lethality. The number of liveborns was 67% of the expected value, but very few survived to weaning age (Table 2). Additionally, hemizygosity for all the gene traps in the \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) background (\( \text{Mcm2}^{\text{Gt1}}, \text{Mcm6}^{\text{Gt}} \) and \( \text{Mcm7}^{\text{Gt1}} \)) caused growth retardation of survivors. The decreased in male weight was \(~50\%\), \(~20\%\) and \(~80\%\) respectively, compared to \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) siblings (Fig. 5) at the oldest age measured (\( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) \( \text{Mcm7}^{\text{Gt1+}} \) animals died before wean, so the oldest weights were taken at 10 dpp. These results demonstrate that incremental perturbations in replication licensing have deleterious consequences in the whole mouse.

To determine if hemizygosity of \( \text{Mcm2} \) would impact survival or tumor susceptibility in animals homozygous for the \( \text{Mcm4}^{\text{Chaos3}} \) allele, mice of various genotypes from the aforementioned cross were aged. \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) \( \text{Mcm2}^{\text{Gt1+}} \) mice (a cohort of 26) began dying at 2 months of age, and all were dead (or sacrificed when they appeared moribund) by 7 months of age (Fig. 6). Necropsies revealed lymphomas or apparent leukemias in 13 of these animals, 5 had enlarged spleens, 3 could not be recovered for proper analysis, and 4 had no clear cause of death. Consistent with previous studies (Shima et al, 2007a), most \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) mice hadn’t yet succumbed from tumors or other causes by 12 months of age. These data show clearly that removing a half dose of MCM2 from \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) cells is sufficient to produce greatly elevated cancer predisposition. I used qRT-PCR to determine that MEFs of this genotype have 47.4% the amount of \( \text{Mcm2} \) mRNA as \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) cells, (data not shown), which already had a 38% reduction compared to WT (Fig. 2). Thus, \( \text{Mcm2} \) RNA levels were reduced to about 29% of WT. To determine if elevated GIN might be responsible for the cancer susceptibility phenotype, we measured erythrocyte micronucleus levels. Whereas the percentage of micronucleated RBCs in \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) mice was 3.73 \( \pm \) 0.394 (mean\_SEM, N=7), \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) \( \text{Mcm2}^{\text{Gt}+} \) mice averaged 5.76 \( \pm \) 0.87 (N=7), indicating a synergistic increase (\( P=0.033 \)).

**Key Research Accomplishments**

- Decreased MCM2-7 in \( \text{Mcm4}^{\text{Chaos3/Chaos3}} \) cells is due to lower Mcm2-7 mRNA levels, which are regulated posttranscriptionally

- Decreased Mcm gene dosages cause elevated chromosomal instability in mice
MCM2-7 are essential for viability of all invertebrate species investigated, and I confirmed that this is true in mammals (note: Mcm5 was not studied).

Heterozygosity for $\text{Mcm2, Mcm6 or Mcm7}$ causes partial synthetic lethality, severe growth defects and (for $\text{Mcm2}$) early tumor predisposition in $\text{Mcm4}^{\text{chaos3/chaos3}}$ mice.

A quantitative MCM threshold is required for embryonic viability, as demonstrated by the synthetic lethalities I observed when combining homozygosity of $\text{Mcm4}^{\text{chaos3}}$ with $\text{Mcm2}^{\text{Gil}}, \text{Mcm6}^{\text{Gil}}$ or $\text{Mcm7}^{\text{Gil}}$ heterozygosity, but not in the heterozygous single mutants. Whatever the exact mechanistic cause of these phenotypes, it is clear that the phenotypes are related to reduction of one or more MCMs below a threshold level that is somewhere below 50%.

Reportable Outcomes:

Manuscript:
Synthetic lethality, genomic instability, and cancer in mice associated with coordinate posttranscriptional downregulation of Mcm genes. Chen-Hua Chuang, Marsha Wallace and John C. Schimenti* Oncogene (in revision).

Student Work-In-Progress Presentations:
Synthetic lethality, genomic instability, and cancer in mice associated with coordinate posttranscriptional downregulation of Mcm genes. Chen-Hua Chuang and John C. Schimenti. Field of Molecular and Integrative Physiology Student Work-In-Progress. Mar 2009 (oral presented)

Meeting Presentations:
Synthetic lethality, genomic instability, and cancer in mice associated with coordinate posttranscriptional downregulation of Mcm genes. Chen-Hua Chuang, Marsha Wallace and John C. Schimenti The Fifth Annual Center for Vertebrate Genomics Symposium. July 2009 (poster presented)

Coordinate downregulation of $\text{Mcm2-7}$ RNAs in mouse cells bearing a cancer-predisposing allele of Mcm4. Chen-Hua Chuang, Marsha Wallace and John C.
Schimenti*  Eukaryotic DNA Replication & Genome Maintenance Meeting  . Cold Spring Harbor Laboratory NY. September 2009. (poster presented)

Admission-to-Candidacy examination:
Synthetic lethality, genomic instability, and cancer in mice associated with coordinate posttranscriptional downregulation of Mcm genes. Chen-Hua Chuang. Dec 2008  (oral and proposal presented)

Conclusions

Defects in DNA metabolism, such as mutations causing replication stress, can lead to genomic instability (GIN). Previous studies in cell culture systems have shown that genetic or siRNA-mediated depletion of the MCM2-7 (minichromosome maintenance) DNA replication licensing factors, which form the replicative helicase, can cause GIN and cell proliferation defects. To explore the effects of incremental attenuation of MCM licensing in whole animals, we generated and analyzed the phenotypes of mice hemizygous for Mcm2, 3, 4, 6, and 7, individually and in combination with each other and the hypomorphic Mcm4Chao3 cancer susceptibility allele. Compound heterozygotes generally exhibited higher GIN than individual heterozygotes. Nullizygosity was lethal in all cases. Synthetic lethality and growth defects were observed in Mcm4Chao3/Chao3 mice that were also heterozygous for disrupted alleles of Mcm2, 6, or 7. Finally, surviving Mcm4Chao3/Chao3, Mcm2+/− animals died of highly penetrant, early onset cancer. Interestingly, Mcm4Chao3/Chao3 embryonic fibroblasts exhibited a ~40% reduction in all MCM proteins that was paralleled by reduced levels of Mcm2-7 mRNA. Studies of hnRNA, RNA Pol II occupancy, and Mcm promoter-reporter assays revealed that the mRNA decreases occur posttranscriptionally. These data suggest that proper stoichemistry of MCM components is controlled at the post-transcriptional level, and that relatively minor disregulation or destabilization of MCM levels can have serious consequences for survival, health, and cancer susceptibility of whole animals.
References:


Supporting Data

Figure 1. Nuclear and cytosolic MCM2-7 proteins are reduced in *Mcm4*<sup>Chao3/Chao3</sup> MEFS. (A) Western blot analysis of MCM2-7. Cytosolic or chromatin-bound protein. The bands correspond to the predicted molecular weights of these proteins. TBP = TATA box binding protein. (B) Quantification of Western blot data. The Western blots were scanned, and densitometry was performed. The amounts relative to WT cells (after normalization to the controls) are plotted. Error bars represent SEM, derived from 4 replicate experiments.

Figure 2. Mcm2-7 mRNAs are reduced in *Mcm4*<sup>Chao3/Chao3</sup> cells. qRT-PCR analysis of (A) Mcm mRNAs and (B) control genes, in the three indicated genotypes of MEFS. Relative transcript levels were normalized to b-actin. Charted are the percent levels of the indicated RNAs in mutant compared to WT (considered to be 100%). At least 3 replicate cultures were analyzed for each genotype. Error bars are SEM.

Figure 3. Depletion of Mcm2-7 mRNAs in *Mcm4*<sup>Chao3/Chao3</sup> cells occurs posttranscriptionally. (A) Mcm2-7 hnRNA levels in *Mcm4*<sup>Chao3/Chao3</sup> MEFS are unchanged compared to WT. Plotted are qRT-PCR data (% compared to WT), of intron/exon amplimers produced with primers listed in Supplemental Table 2. N = 3 replicates; SEM bars are shown. (B) ChIP-qPCR analysis of RNA Pol II occupancy within the Mcm2-7 transcription units of *Mcm4*<sup>Chao3</sup> mutant MEFS. N = 4 replicates; SEM bars are shown. (C) Luciferase reporter assays. Plotted are the luciferase activities of in *Mcm4*<sup>Chao3/Chao3</sup> MEFS transfected with the indicated promoter-luciferase (Luc) expression constructs (see Methods), with the values relative to transfections into WT MEFS. N = 5 replicates; SEM bars are shown.
Figure 4. Increased GIN and variable mRNA reductions in mice with Mcm mutations. (A) Micronuclear levels in Mcm gene trap-bearing male mice. All of the Mcm gene trap-bearing mice are grossly normal. At least 5 animals were analyzed for each single gene trap mutant allele. The “2GT” (two gene trap) group contains: 4 mice doubly heterozygous for Mcm2<sup>GT</sup> and Mcm3<sup>GT</sup> (“Mcm2/3”), 4 Mcm2/4 mice, and 4 Mcm3/4 mice. The 3GT group contains: 4 Mcm2/3/4 mice, 1 Mcm2/3/6 mouse, 1 Mcm2/4/6 mouse, and 3 Mcm3/4/6 mice. The 4-5GT group contains: 3 Mcm2/3/4/6 mice, 1 Mcm2/3/6/7 mouse, and 2 Mcm2/3/4/6/7 mouse. SEM bars are shown. (B) qRT-PCR analysis of mRNAs in the indicated genotypes of MEFs. Relative transcript levels were normalized to b-actin. Charted are the percent levels of the indicated RNAs in mutant compared to WT (considered to be 100%). At least 3 replicate cultures were analyzed for each genotype.

Figure 5. Effect of Heterozygosity for Mcm2 (a), Mcm6 (b), Mcm7 (c) in Mcm4<sup>Chaos2/Chaos3</sup> mice on body weight. Body weight was measured timely. The number of mice was between 4 and 10 per group. The decreased in male weight was ~50%, ~20% and ~60% respectively, compared to Mcm4<sup>Chaos2/Chaos3</sup> siblings.

Figure 6. Premature morbidity and cancer susceptibility in Mcm<sup>Chaos2/Chaos3</sup> Mcm2<sup>GT/+</sup> mice. Kaplan-Meier survival plot of the indicated genotypes. As indicated, all but 1 (representing both sexes) of the Mcm<sup>Chaos2/Chaos3</sup> Mcm2<sup>GT/+</sup> animals have died. Abbreviations: Mcm<sup>GT</sup> = Mcm4<sup>Chaos3</sup>. 
Table 1. Mcm3<sup>genotype</sup> is abbreviated as Mcm<sup>OT</sup>. The chi<sup>2</sup> P value for all Cross relates to deviation of all classes in toto from the expected.

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<th>Cross 1: Mcm2&lt;sup&gt;OT&lt;/sup&gt; X Mcm2&lt;sup&gt;OT&lt;/sup&gt;</th>
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Table 2. Mcm4<sup>Cross3</sup> is abbreviated as Mcm4<sup>C3</sup>. The chi<sup>2</sup> P value for Cross1 relates to deviation of all classes in toto from the expected. In Cross 2, the P value is derived from the Fisher’s Exact Test (FET) in comparing the Mcm4<sup>C3</sup>C3 Mcm2<sup>OT</sup>/+ (shaded for emphasis) vs. the Mcm4<sup>C3</sup>C3 classes. In Cross 7, the chi<sup>2</sup> value addresses numbers at birth, but as indicated, most died by weaning age.